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It is clear that even though it is well established in the scientific community that periodontal disease is a multi-factorial disease no one has yet developed a test to analyse both bacterial and host derived products in a sample.

The test kit may also comprise a third detection assay comprising at least a third affinty ligand having a binding site for binding of a third substance originating from bacteria or from the immune or inflammatory system of the patient as defined above, preferably from bacteria, and may in some embodiments even comprise further detection assays for binding of additional substances.

Preferably, the first affinity ligand is an antibody exhibiting selective binding of said first substance, and the second affinity ligand is an antibody exhibiting selective binding of said second substance.

The use of antibodies is advantageous over other methods (e.g. enzymatic methods) since antibodies, once developed and tested for cross-reactivity, are highly specific for their target and are able to detect chemical substances other than enzymes, e.g. toxins and cytokines. Furthermore, methods for development of new antibodies against new antigens are well known to those skilled in the art.

Preferably said detection assays in the test kit according to the present invention comprises immunochromatographic assays.

Among the advantages of using immunochromatographic assays/methods is that they are easily produced and used, have a long shelf-life, yields a quick answer and can be designed to be very specific for the substances intended to be detected.

Said test kit further preferably comprises a support provided with a sample reservoir for receiving a sample, wherein said first and second detection assays are arranged on said support in contact with said sample reser-

oides forsythus or a toxin, more preferably the leukotoxin from *Actinobacillus actinomycetemcomitans*.

The presence of virulence products may trigger an inflammatory response and the immune system of the host, thus recruiting defence system cells, e.g. polymorphonuclear (PMN) leukocytes, to the site of infection.

The leukocytes cannot cope with the high amounts of bacteria and bacterial products at the infected site and enzymes from the leukocyte granulates are released into the periodontal pocket. These enzymes, originally aimed at killing the invading bacteria, are highly destructive to the surrounding tissues. Human neutrophil elastase has been shown to degrade many of the supportive tissues surrounding a tooth. Elastase is often found bound to its protease inhibitor (α-1 antitrypsin) in the periodontal pocket. However, the protease from *P. gingivalis* has been shown to degrade the protease inhibitors α-1 antitrypsin and α-2 macroglobulin in human serum, leaving elastase in its highly destructive form in the periodontal or peri-implant pocket.

As used herein, substances "originating from the immune or inflammatory system" refers to substances that originates from cells involved in the immune or inflammatory system. Such substances may be secreted from said cells, or may originate from lysis of such cells, for example to orchestrate the immune and inflammatory response or to remodell the tissue or killing the invading bacteria.

The second substance may be a leukocyte product, such as a natural serine protease, preferably human neutrophil elastase or a cytokine, such as an interleukin preferably chosen from among interleukin-1β, interleukin-6 and interleukin-8, or an inflammatory mediator, preferably tumour necrosis factor-α or possibly prostaglandin E_2.

Most preferably, said second substance is human neutrophil elastase.

The invention also relates to a method for diagnosing periodontal diseases and/or predicting the risk for progress of said diseases, said method comprising analyzing a sample from the oral cavity of a patient for the presence of at least a first substance originating from bacteria and the presence of a second substance originating from the immune or inflammatory system of the patient.

In the method according to the present invention, sample from the oral cavity of a patient and the first and second substances are as defined above.

Also in some instances, the diagnostic method according to the present invention further comprises the step of detecting the presence of additional substances as defined above in said sample.

In preferred embodiments of the diagnosis method according to the present invention, said first method comprises using a first antibody exhibiting selective binding of said first substance and said second method comprises using a second antibody exhibiting selective binding of said second substance, wherein said antibodies are as defined above.

Most preferably at least one of said first and second methods comprises using an immunochromatographic method.

Other detection methods suitable for use with the methods according to the present invention may be based on one out of several immunological methods. Such methods include, but are not limited to, immunochromatographic methods, immunometric methods, immunoagglutination methods, fluoroimmunological methods, immunoluminescence methods, turbidimetric immunolological methods, ELISA and nephelometric methods.

Preferred embodiments

Preferably a test kit according to the invention, as illustrated in figure 1, comprises two immunochroma-

tographic detection assays 1, 2 arranged on a support 3 equipped with a sample reservoir 4 for receiving a sample. The two detection assays 1, 2 are arranged, directly or via a removably arranged separating means 5, in contact with the sample reservoir 4.

The sample reservoir 4 is preferably formed in the support material. The support 3 can be made of several different material, such as plastic, paper, carton or combinations thereof, such as paper laminated with plastic. The detection assays 1, 2 are fixed on the support 3 in such a manner that the sample receiving areas of each assay is, directly or via a removably arranged separating means 5, in contact with the sample reservoir 4. Said separating means 5 may be a removable foil covering the sample receiving areas on the assays, a dam separating the reservoir from the assays, etc.

The kit may further comprise additional buffers for dilution and adaptation of said sample for said detection assays and at least one sampling device for obtaining the sample to be analysed. The type of sampling device suitable for use may depend on the type of sample to be analysed. For example gingival crevicular fluid is a viscous fluid and can easily be collected by a small brush, a dental floss, a paper point or a disposable pipette. Other sampling devices for obtaining a saliva or mouth rinse sample are known to those skilled in the art. Preferably the buffer is kept separately from the sample reservoir in a buffer reservoir, such as a separate flask, an additional reservoir formed on the support or by providing the buffer in a puncturable bag placed in the sample reservoir.

After a sample is obtained, it is preferably mixed with buffer to obtain the pH, ionic strength and viscosity suitable for the detection assays. After mixing, the sample is transferred to the sample reservoir, and is thus, optionally by removing the separating means,

(Nunc, Rosklide, Denmark) containing 100 µl 0,85% NaCl. The samples were frozen immediately at -20°C and within 6 hours at -80°C, and stored until assayed. For the microbial analysis 3 paper points were inserted consecutively into the periodontal pocket until resistance was met and left in place for 15 secondes. The points were pooled into a vial containing 10 glass beads, 3 mm in diameter, and 3,3 ml of VMGA III transport medium, aerobically prepared and stored. The samples were processed within 24 hours. Bacteria were grown on enriched Brucella agar plates and identified by appropriate methods.

For the study of appropriate cut-off values for a diagnostic test we studied elastase from human neutrophils and arg-gingipain from *P. gingivalis* using selective substrates.

The first set of the duplicate samples was used for the enzyme assays. All samples were thawed on ice and centrifuged for 3 minutes at 13,000 × g.

The enzyme substrate for determining arg-gingipain from *P. gingivalis* was N-benzoyl-L-arginine-p-nitroanilie (BAPNA) with a final concentration of 1 mM in the assay buffer containing 5 % DMSO. The assay buffer was 0.1 M Tris-HCl containing 5 mM CaCl, pH 7,5, with 50 mM glycyl-glycine (as reported earlier by the inventors in patent US 5,981,164 gly-gly stimulates BAPNA selectively in the presence of arg-gingipain) and 5 mM L-cysteine. Ten µL of the sample was preincubated for 15 minutes with 140 µL of the assay buffer in the well of a 96-well microtiter plate, precoated with bovine serum albumin, before 50 µL of the substrate was added. The plate was incubated at 37°C in a humid chamber, and the release of pNA was followed spectrophotometrically by OD_{405} readings, using a microtiter plate reader, every few hours from 12 to 36 hours. One unit of activity was equal to the amount of enzyme which cleaved 1 nmol of the substrate during one hour of incubation.

Results

Elastase		Attachment loss	Attachment gain or zero
Positive test (Elastase >20 ng)		3	2
Negative test (Elastase ≤20 ng)		3	27

Elastase as a predictor of further attachment loss yielded a sensitivity of 50% and a specificity of 93%. A p-value of 0.0264* was calculated using Fisher's exact test.

Arg-gingipain		Attachment loss	Attachment gain or zero
Positive test (arg-gingipain >0,27 U)		6	13
Negative test (arg-gingipain ≤0,27 U)		0	16

Arg-gingipain as a predictor of further attachment loss yielded a sensitivity of 100% and a specificity of 55%. A p-value of 0.0216* was calculated using Fisher's exact test.

For elastase and arg-gingipain alone either the senistivity or the specificity had to be sacificed. This promted us to investigate the combination of the two enzymes. We investigated new cut-off levels for the two enzymes to find that the detection limit for elastase should be 2 ng per site and 0,30 units for arg-gihipain.

Combination		Attachment loss	Attachment gain or zero
Positive test (Elastase >2 ng and arg-gingipian >0,30 U)		5	3
Negative test (Elastase ≤2 ng or arg-gingipain ≤0,30 U)		1	26

The combination of elastase and arg-gingipain as a predictor of further attachment loss yielded a sensitivity of 83% and a specificity of 90%. A p-value of less than 0,0001*** was calculated using Fisher's exact test. This limited data shows that the combination of elastase and arg-gingipain as a marker for periodontal diesease yields a statistically more significant test than either of the enzymes alone.

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CLAIMS

1. A test kit for detecting periodontal disease in a patient by analysing a sample from the oral cavity of the patient, wherein said kit at least comprises:

a first detection assay for detecting a first substance originating from bacteria, and

a second detection assay for detecting a second substance originating from the immune or inflammatory system of the patient.

2. A test kit according to claim 1, wherein said first detection assay comprises at least a first affinity ligand having a binding site for binding said first substance originating from bacteria, and

said second detection assay comprises at least a second affinity ligand having binding site for binding said second substance originating from the immune or inflammatory system of the patient.

3. A test kit according to claim 1 or 2, wherein said first substance is a bacterial virulence product.

4. A test kit according to claim 3, wherein said first substance is an enzyme.

5. A test kit according to claim 4, wherein said enzyme is a protease.

6. A test kit according to claim 5, wherein said protease is selected from the group consisting of arggingipain from *Porphyromonas gingivalis* and a 48 kDa protease from *Bacteroides forsythus*.

7. A test kit according to claim 3, wherein said first substance is a toxin.

8. A test kit according to claim 7, wherein said toxin is a leukotoxin from *Actinobacillus actinomycetemcomitans*.

9. A test kit according to any of the preceding claims, wherein said second substance is a leukocyte product.

10. A test kit according to claim 9, wherein said leukocyte product is a natural serine protease.

11. A test kit according to claim 10, wherein said natural serine protease is a human neutrophil elastase.

12. A test kit according to any of the claims 1-8, wherein said second substance is a cytokine.

13. A test kit according to claim 12, wherein said cytokine is an interleukin.

14. A test kit according to claim 13, wherein said interleukin is chosen from among interleukin-1β, interleukin-6 and interleukin-8.

15. A test kit according to claim 12, wherein said cytokine is an inflammatory mediator.

16. A test kit according to claim 15, wherein said inflammatory mediator is selected from the group consisting of tumour necrosis factor-α and prostaglandin E_2.

17. A test kit according to any of the claims 2 to 16, wherein said first affinity ligand is a first antibody exhibiting selective binding of said first substance and said second affinity ligand is a second antibody exhibiting selective binding of said second substance.

25. A method according to claim 24, wherein said first substance is a bacterial virulence product.

26. A method according to claims 25, wherein said first substance is an enzyme.

27. A method according to claim 26, wherein said enzyme is a protease.

28. A method according to claim 27, wherein said protease is selected from the group consisting of arg-gingipain from Porphyromonas gingivalis and a 48 kDa protease from Bacteroides forsythus.

29. A method according to claim 25, wherein said first substance is a toxin.

30. A method according to claim 29, wherein said toxin is a leukotoxin from Actinobacillus actinomycetemcomitans.

31. A method according to any of the claims 24-30, wherein said second substance is a leukocyte product.

32. A method according to claim 30, wherein said leukocyte product is a natural serine protease.

33. A method according to claim 32, wherein said natural serine protease is a human neutrophil elastase.

34. A method according to any of the claims 24-30, wherein said second substance is a cytokine.

35. A method according to claim 36, wherein said cytokine is an interleukin.

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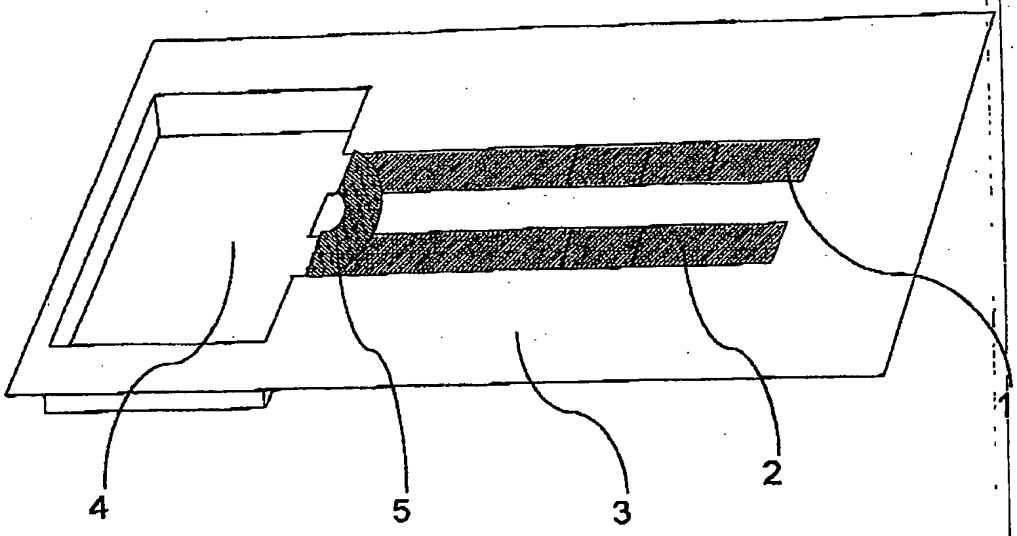

Fig. 1